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(54) Title: ENHANCED CRYOPRESERVATION WITH THERMAL HYSTERESIS PEPTIDE (57) Abstract A composition is provided for enhancing survival of biological materials during freezing and thawing. The composition in- cludes at least one cryoprotectant and a thermal hysteresis peptide in an amount effective for enhancing the viability of the bio- logical material. A method of enhancing survival of biological materials during freezing and thawing is also provided. A method for preserving the functional integrity of membrane enclosed of bound material during freeze-drying is also provided.		

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- 1 -

DescriptionENHANCED CRYOPRESERVATION WITH THERMAL HYSTERESIS PEPTIDE

This application is a continuation-in-part of U.S. Serial No. 07/486,822, filed March 1, 1990, still pending, which is hereby expressly incorporated by reference.

BACKGROUND OF THE INVENTION

The present invention relates to a method for increasing the percentage of cells remaining viable after cryopreservation and storage at ultracold temperatures and thawing. That is, the process described herein results in an enhancement of the preservation capacity provided by classical cryoprotectants.

According to certain advantageous features, the present invention allows one to use a lower concentration of classical cryoprotectant (e.g., dimethylsulfoxide) and still achieve the same level of cell viability after thawing. In addition, with certain cryoprotectants (e.g., polyvinylpyrrolidone and hydroxyethyl starch), the use of the process described herein results in higher viability than is achievable when the cryoprotectant is used alone. Furthermore, the present invention minimizes the damage arising due to suboptimal warming rates and hence allows one more leeway in thawing samples.

Current medical technology allows the use of several different types of tissue and cells for transplantation and/or transfusion to correct congenital, disease- or trauma-induced, or degenerative failure of a recipient's cells or tissues. Some examples include cells and allograft human heart valves, veins, corneas, bone marrow, etc.

Cryopreservation and ultracold storage of cells and tissue became possible after the discovery in 1949, by Polge, Smith and Parks, that glycerol could be used to protect cells from injury due to freezing. Workers in the medical and biological fields have been seeking better ways to

- 2 -

cryopreserve cells and to circumvent some of the drawbacks of classical cryopreservation regimes (e.g., toxicity of cryoprotectants at physiological temperatures and need to thaw samples very rapidly).

Several methods for freezing cells and tissues have been reported. For example, U.S., Patent No. 3,303,662 refers to a process for cell preservation that utilizes a cryoprotectant in the freezing process. One drawback of many cryopreservation protocols is that at high concentrations the cryoprotective agent can be toxic to cells. For example, more than 80% of fetal pancreas cells remain viable after freeze-thawing in the presence of 2M dimethylsulfoxide, but less than 50% recovery is noted with 3M dimethylsulfoxide (Mazur, In "Organ Preservation for Transplantation," 2nd edition, pp. 143-175, Mariel Deker, New York, 1981). (see Fahy, Cryobiology, 23:1-13, 1986). Therefore, it would be advantageous to develop methods that allowed for an equal level of cell preservation with a reduced amount of cryoprotectant.

In addition, an inherent component of most cryopreservation procedures is the formation of ice crystals. Intracellular ice formation and crystal growth is usually thought to be lethal to cells. Even extracellular ice can cause damage to frozen cells and tissue, especially if they are thawed at a slow rate, which allows migratory recrystallization (the increase in ice crystal size as the sample is warmed from ultracold temperatures). The growing crystals can cause direct mechanical disruption of the tissue or cellular integrity. Finally, even when cells or tissues are frozen in such a manner that ice crystals do not form (i.e., vitrification), devitrification and ice crystal growth during thawing can be damaging.

The nature and extent of ice formation during cooling plays an important role in the survival of cells during freeze-thawing (See Mazur, Organ Preservation for

- 3 -

Transplantation (Karow et al., editors) (various cells), pp. 143-175 (1981); Pegg et al., Cryobiology 21:491 (1984) (red blood cells)). In certain situations, rapid thawing has been required to prevent growth of large ice crystals which can result in damage to the biological material. In addition, it has been shown that the propagation of large ice crystals at the expense of smaller crystals during warming also contributes to cellular damage (Pegg, et al., 1984). For example, this has been shown with red blood cells. It has been demonstrated that slowly warming ($0.3^{\circ}\text{C}/\text{min}$) solutions of red blood cells, which had previously been cooled to -100°C (in the presence of 2 M glycerol), resulted in a massive increase in the crystal size of the extracellular ice and greater than 50% hemolysis (Pegg, the Biophysics of Organ Preservation (Pegg et al., editors), pp. 117-136 (1987)). In contrast, hemolysis was less than 10% for preparations warmed at $100^{\circ}\text{C}/\text{min}$, in which ice simply melted instead of recrystallizing (Pegg). The prevalence of recrystallization, either alone or following devitrification, during slow warming probably accounts for the fact that certain cells frozen in certain cryoprotectants must be warmed very rapidly to realize preservation of a high percentage of cells. This is shown, for example, with red blood cells frozen in hydroxyethyl starch (Weatherbee, DOD Technical Report AD-A018 387 (1975); Lionetti, et al. DOD Technical Report AD-A020 513 (1975)).

The present inventor has surprisingly discovered that certain naturally-occurring molecules and derivatives thereof provide enhancement of cell survival during cryopreservation and thawing. These molecules are called thermal hysteresis peptides (THPs). THPs are found in both polar fish and insects (DeVries, Phil. Trans. R. Soc. Lond. B 304:575, 1984; Knight and Duman, Cryobiology 23:256, 1986).

These peptides have also been characterized as "antifreeze peptides (AFP)" or "antifreeze glycopeptides

- 4 -

(AFGP)." It has been known for many years that fish have been able to inhabit the ice-laden waters of the polar oceans. However, only recently has it been learned that survival of these fish in such freezing environments is largely due to the presence in the serum of macromolecular ice crystal control peptides and glycopeptides, which depress the freezing temperatures of the body fluids. These ice crystal control materials reduce or prevent ice crystal growth, and have also been characterized as antifreezes. Four classes of these fish macromolecular antifreezes have been identified to date.

The first class of macromolecular antifreezes, found in Nototheniids and Gadoids, are antifreeze glycopeptides. These range in molecular weight from 2,600 to 37,000 and contain high proportions of alanine and threonine with a disaccharide moiety covalently linked to the threonine residues. The second, third and fourth classes of antifreezes are peptides, and are referred to in Hew et al., Journal of Chromatography, 296:213-219 (1984). The second class of antifreezes are small peptides, molecular weight 3,300 to 4,000, which also show a high alanine content but have no carbohydrate attached. These are found in winter flounder and shorthorn sculpin. The third class is a 9,900 molecular weight peptide found in sea raven. It also contains no carbohydrate but unlike all other known antifreezes it contains cysteine (7.6%). The fourth class is isolated from ocean pout, has a molecular weight of approximately 6,000 and is neither alanine-rich nor cysteine-rich.

The macromolecular peptides and glycopeptides show freezing point depression in a non-colligative manner. Thus, the peptides and glycopeptides lower the freezing temperature of water but have little to no effect on its melting temperature. Hence, the term "hysteresis" of the term "THP"

- 5 -

means that there is the inhibition of freezing with little to no effect on melting.

The capacity to depress freezing point per se is not beneficial during cryopreservation procedures, since at concentrations around 40 mg/ml (usually the highest level tested in published reports) the THPs depress freezing point only to about -1.2°C . For most applications, cryopreserved samples are cooled to and stored at the temperature of liquid nitrogen (-196°C) or that of liquid nitrogen vapor .

(about -130°C). It has also been found that antifreeze glycopeptides at concentrations of about 10^{-7} to 10^{-10} M effect the morphology of growing ice crystals.

Knight et al., Nature, 308:295-296, 1984. Peptide antifreeze isolated from winter flounder also produces freezing hysteresis and similarly effects ice growth. Knight et al., at page 296.

These peptides also have been shown to inhibit the recrystallization of ice under isothermal conditions and at annealing temperatures no lower than -8°C . Knight et al., Cryobiology 23: 256-262, 1986; Knight et al., Cryobiology 25:55-60, 1988. Those experiments do not suggest the use of such protein for recrystallization inhibition during cryopreservation to ultracold temperatures. First, the temperature range used during cryopreservation is much lower than that used by Knight et al. to characterize recrystallization inhibition by THPs. Second, during freezing and thawing, cryopreserved samples are subjected to changing temperatures and are not held isothermally for long time periods at high subzero temperatures (temperatures above -130°C).

In certain initial studies, the present inventors noted that the presence of THP induced damage to cells during freeze-thawing and negated the protective effects of cryoprotective compounds such as glycerol and dimethylsulfoxide. However, in subsequent studies, the

SUBSTITUTE SHEET

- 6 -

present inventors surprisingly discovered methods by which THPs could be used to enhance the level of cryopreservation provided by cryoprotectants.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method of enhancing the survival of cryopreserved biological material, such as tissues and cells, upon thawing.

It is yet another object of the present invention to utilize thermal hysteresis proteins to effect this increase in cell survival by enhancing the degree of cryopreservation provided by penetrating and extracellular cryoprotectants.

It is yet another object of the present invention to use thermal hysteresis proteins to reduce the concentration of a cryoprotectant that is needed to achieve a given degree of protection, and thus, reduce the possibility of cryoprotectant toxicity.

It is yet another object of this invention to use thermal hysteresis proteins to increase the degree of preservation to a higher level than can be achieved by even the most effective concentration of a given cryoprotectant, when used without THPs.

It is yet another object of this invention to use thermal hysteresis peptide, to minimize the damage to biological material arising during thawing, thus allowing one to thaw samples at suboptimal warming rates and still achieve a high degree of cell survival.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may be learned from practice of the invention. The objects and advantages may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

SUBSTITUTE SHEET

- 7 -

These and other objects, features, and advantages of the present invention are achieved by providing a method for enhancing survival of biological material during cryopreservation and thawing, which is comprised of freezing and thawing biological material in the presence of a penetrating or nonpenetrating (extracellular) cryoprotectant and a thermal hysteresis peptide.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate various embodiments of the invention and, together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 compares the survival of human KG-1A cells after freeze-thawing in the presence of thermal hysteresis peptide alone and in the presence 5% (vol/vol) dimethylsulfoxide in combination with thermal hysteresis peptide. Samples were cooled at 1°C/min, stored in liquid nitrogen vapor, and thawed in a 37°C water bath. Each point represents the mean \pm SE for six replicates.

Fig. 2 shows the effect of thermal hysteresis peptide on the survival of human KG-1A cells that were cooled at 5°C/min in the presence of 5% (vol/vol) dimethylsulfoxide, stored in liquid nitrogen vapor, and thawed in air at room temperature. Each point represents the mean \pm SE for six replicates.

Fig. 3 shows the effect of thermal hysteresis peptide on the survival of human KG-1A cells that were cooled at 1°C/min in the presence of 12% (vol/vol) glycerol, stored in liquid nitrogen vapor, and thawed in a 37°C water bath. Each point represents the mean \pm SE for six replicates.

- 8 -

Fig. 4 shows the effect of thermal hysteresis peptide on the survival of human KG-1A cells cooled at 3°C/min in the presence of 20% (wt/vol) of either polyvinylpyrrolidone or Ficoll. Samples were stored in liquid nitrogen vapor and thawed in a 37°C water bath. Each point represents the mean \pm SE for six replicates.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the following examples, serve to explain the principles of the invention. All references cited herein are hereby incorporated by reference.

The following amino acids are abbreviated herein as follows:

Ala is Alanine,
Arg is Arginine,
Asn is Asparagine,
Asp is Aspartic acid,
Gln is Glutamine,
Glu is Glutamic acid,
His is Histidine,
Ile is Isoleucine,
Ser is Serine,
Thr is Threonine, and
Val is Valine.

The present invention encompasses a method for enhancing cell and tissue, or other biological material, survival during cryopreservation and thawing, which is comprised of freezing and thawing biological material such as cells, tissues and/or organs in the presence of a penetrating or nonpenetrating cryoprotectant(s) and a thermal hysteresis peptide. In the field of cryopreservation of biological materials, the goal is to maximize the viability and functionality that is present upon thawing.

SUBSTITUTE SHEET

- 9 -

To achieve this goal, often it is desirable to reduce the level of cryoprotectant used to preserve cells, tissues or organs since many of the classical penetrating cryoprotectants are toxic to cells at physiological temperatures as discussed above. (Fahy, Cryobiology 23:1-13, 1986). In addition, some cryoprotectants are more preferable because they are less toxic and/or pose less of an osmotic stress to cells during freeze-thawing regimes, for example, nonpenetrating cryoprotectants such as polyvinylpyrrolidone and hydroxyethyl starch. However, these compounds when used alone often do not provide a sufficient level of cryoprotection to cells. Moreover, often extremely rapid thawing is needed to achieve cell survival during freeze-thawing with these compounds. By the use of combinations of thermal hysteresis peptide and cryoprotectants, the present invention provides a novel and surprising solution to these long-standing problems in cryobiology.

As used herein, the term "thermal hysteresis peptide (THP)" refers to a class of macromolecular ice crystal control peptides and glycopeptides. They are referred to as thermal hysteresis molecules or antifreezes because they lower the freezing point of water without altering the melting point significantly. These ice crystal control materials also reduce or prevent migratory ice crystal growth, a process known as recrystallization. THPs are found in nature in certain polar fish, winter flounder and in certain freeze-tolerant insects.

The discoveries described by the present invention were based on research with THP from the winter flounder, which has a molecular weight of 3300, is comprised of 37 amino acids and has the following formula: (NH_2) ASP-THR-ALA-SER-ASP-ALA-ALA-ALA-ALA-ALA-ALA-LEU-THR-ALA-ALA-ASN-ALA-LYS-ALA-ALA-ALA-GLU-LEU-THR-ALA-ALA-ASN-ALA-ALA-ALA-ALA-ALA-ALA-THR-ALA-ARG (COOH) . The protein was produced by use of a

- 10 -

synthetic gene and was expressed in the bacterium E. coli, as described in patent application serial no. 07/394,881. However, the methods and compositions described herein are applicable to any of the THP molecules derived by purification from natural sources, chemical synthesis using solid phase techniques or synthesis by use of recombinant gene technology, which molecules demonstrate the properties of controlling ice crystal growth.

The term ultracold temperatures is defined as . temperatures lower than -100°C . Room temperature is a term known in the art and according to certain preferred embodiments encompasses temperatures of 20°C to 24°C .

The term "cryoprotectant", as used herein, refers to broad groups of organic molecules that have been shown to protect cellular viability and function, the morphological and functional integrity of tissues and their component cells, and the structure and function of biomaterials, for example, liposomes and isolated enzymes, during freezing and storage at ultracold temperatures, and thawing. Penetrating cryoprotectants are those molecules that can permeate across the plasma membrane into the interior of cells and examples include dimethylsulfoxide, glycerol, ethylene glycol, propane diols, butane diols, amide compounds (e.g., formamide), and others. Extracellular or nonpenetrating cryoprotectants are those molecules that do not permeate across the plasma membrane and include, for example, relatively large organic polymers such as polyvinylpyrrolidone, hydroxyethyl starch, dextrans, FICOLL by PHARMACIA, polyethylene glycol, and others. Other classes of compounds that may serve as extracellular cryoprotectants include: sugars (e.g., sucrose, maltose and trehalose), amino acids (e.g., proline, glutamate, and glycine), methylamines (e.g., betaine and sarcosine), and polyhydroxy alcohols (e.g., sorbitol and mannitol). The foregoing examples of suitable cryoprotectants are exemplary only, and should not be

- 11 -

considered exhaustive. Enhanced cryoprotection can be realized using the present invention by combining THP with individual cryoprotectants or mixtures of cryoprotectants.

The methods and formulations described herein apply to cells, tissues and organs, as well as other biomaterials that are sensitive to freeze-thawing, but which can be cryopreserved with one or a combination of the compounds listed above. Such noncellular biomaterials include, but are not limited to, liposomes, isolated enzymes, encapsulated enzymes, and cell organelles.

The cell, tissue, organ or noncellular biomaterial is placed in a suitable tissue culture medium, physiological saline or buffer, which contains at least one cryoprotectant in combination with at least one thermal hysteresis peptide. The choice of aqueous medium, as well as the cryoprotectant, will depend on various factors such as the material to be preserved, any constraints on freezing rate (e.g., sample volume and geometry), and sensitivity to cryoprotectant toxicity. The present inventors discovered that the cooling and warming protocols and the concentrations of cryoprotectant and thermal hysteresis peptide must be chosen such that the thermal hysteresis peptide enhances cryoprotection. Without proper matching of these parameters, the thermal hysteresis peptide can actually negate the protective effect of the cryoprotectant and induce damage to the frozen and thawed sample.

The frozen sample can then be stored at ultracold temperatures, for example, in liquid nitrogen (-196°C) or in liquid nitrogen vapor (about -130°C). Alternatively, a mechanical freezer that maintains temperatures below -100°C can be used. An additional advantage of the present invention is that samples can be stored at relatively high subzero temperatures, or temperatures greater than -100°C , for example, -80° or -20°C , and the presence of the thermal

- 12 -

hysteresis peptide will minimize damage that can be induced by migratory ice recrystallization during long term storage.

One further advantage of the present invention is that, regardless of the type of sample to be frozen, the presence of the thermal hysteresis peptide can enhance the degree of preservation provided by the cryoprotectant, when the proper combination of parameters is used.

Also, Example 7, which follows, describes the possible use of THP and lyoprotectants to enhance red blood cell survival during freeze-drying. Although the materials are the same, the term "cryoprotectant" is changed to "lyoprotectant" when such materials are used during freeze-drying rather than during cryopreservation. THP should minimize damage due to ice crystal growth in samples being freeze-dried. Samples processed by this technique are often frozen rapidly, which results in very small ice crystals. During the sublimation of the samples under vacuum, there is opportunity for these ice crystals to increase in size through migratory recrystallization. Such recrystallization has been documented and it could damage cells and cellular elements during freeze-drying. This is analogous to the recrystallization damage that is noted during the thawing portion of a freeze-thawing protocol. THP should inhibit recrystallization during sublimation and thus enhance the viability of freeze-dried products.

The following specific examples will illustrate certain embodiments of the invention applied to the cryopreservation of human cells. However, as described above, it will be appreciated that these teachings apply to all biomaterials, including those containing cells, as well as noncellular samples. Various alternatives will be apparent to those of ordinary skill in the art from the teachings herein, and the invention is not limited to the specific illustrative examples.

- 13 -

EXAMPLE 1

Human bone marrow cells (ATCC CCL 246.1, acute myelogenous leukemia; KG-1A) were centrifuged and resuspended in DMEM (with 10% FCS) at a density of 100,000 cells/ 150 ul. All subsequent steps were carried out on ice. 150 ul aliquots of the cell suspension were pipetted into each of the peripheral wells of a 96-well microtiter plate. To each well was added a 150 ul aliquot of DMEM (with 10% FCS) containing 10% (vol/vol) dimethylsulfoxide (to give a final dimethylsulfoxide concentration of 5%) and 2-times the desired final concentration of thermal hysteresis peptide. The THP used was that described in patent application serial no. 07/394,881 which is produced by use of a synthetic gene expressed in the bacterium *E. coli*. This protein, which is found in nature in winter flounder, has a molecular weight of 3300 and is comprised of 37 amino acids as follows: (NH₂) ASP-THR-ALA-SER-ASP-ALA-ALA-ALA-ALA-ALA-LEU-THR-ALA-ALA-ASN-ALA-LYS-ALA-ALA-ALA-GLU-LEU-THR-ALA-ALA-ASN-ALA-ALA-ALA-ALA-ALA-ALA-ALA-THR-ALA-ARG (COOH).

Samples were held on ice for 0.5 hour, to allow penetration and equilibration of the cryoprotectant, and frozen by cooling at 1°C/min in a programmable freezer. After samples had cooled to -80°C, they were transferred to the vapor phase of a liquid nitrogen storage freezer (about -130°C) and held for at least 16 hours before thawing.

Cell samples were thawed by floating the microtiter plate on 37°C water bath. After thawing, the cells were pelleted by centrifugation and 200 ul of the supernatant was removed. The cryoprotectant was diluted by the stepwise addition of 50, 50 and 100 ul aliquots of DMEM. The cells were repelleted by centrifugation and 200 ul of the supernatant was removed and replaced with 200 ul of DMEM. After this step was repeated, the cells were then assayed for residual viability.

- 14 -

Cell viability was assessed using an assay for mitochondrial function, which is based on the reduction of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to form a blue product. The cells were incubated in the presence of the 0.5 mg/ml MTT for 2 hours at 37°C and the assay was terminated by addition of 40 mM HCl, prepared in isopropanol. The assay was performed directly in the microtiter plates in which the cells were frozen and thawed, and the O.D. of samples were read with a microtiter plate spectrophotometer. MTT reduction by frozen and thawed cells was normalized relative to values for control, nonfrozen cells.

This experiment tested the ability of THP, in concentrations ranging from 0 - 1 mg/ml, to protect frozen thawed cells, both in the presence of 5% (vol/vol) dimethylsulfoxide and without any additional cryoprotectant. The results are presented in Fig. 1. Cell survival was almost undetectable in samples frozen and thawed in the presence of THP alone, which indicates that the peptide itself does not serve as a cryoprotectant.

However, in the presence of 5% dimethylsulfoxide, 1 - 100 µg/ml THP enhanced the level of cryopreservation afforded by DMSO. In contrast, levels of THP \geq 500 µg/ml induce greater cell damage. These results demonstrate that, when used at the optimum concentration, THP can serve to enhance the level of cryoprotection afforded by dimethylsulfoxide. Therefore, to achieve a given level of cryoprotection, assuming that degree of protection correlates directly with the concentration of dimethylsulfoxide, less dimethylsulfoxide would be needed when the THP was used. Alternatively, these findings show that the degree of protection afforded by a given concentration of dimethylsulfoxide (during a given cooling and warming regime) can be maximized by the presence of the proper concentration THP.

- 15 -

EXAMPLE 2

KG-1A cells were prepared and frozen as described in Example 1, except that the cooling rate was 5°C/min. The cell samples were thawed in air at room temperature. Cryoprotectant dilution and viability assessment were performed as described in Example 1. The results, shown in Figure 2, indicate that survival of KG-1A cells frozen and thawed under these conditions is enhanced in the presence of 1-30 µg/ml THP.

EXAMPLE 3

KG-1A cells were prepared and frozen as described in Example 1, except that 12% (vol/vol) glycerol was used as the cryoprotectant and the cell density was 200,000 cells/ 150 µl. The cell samples were thawed in a 37°C water bath and the cryoprotectant was diluted as described in Example 1. Viability was assessed by measuring the incorporation of tritiated thymidine by cells. Using the thymidine incorporation assay, the effects of freeze-thawing on overall cell viability and replication are tested. Cells, in microtiter plates, were incubated for 24 hours in the presence of 1 µCi/ml ³H thymidine and then harvested and washed using a cell harvester. The resulting filter discs containing cells were then assayed for tritium counts by liquid scintillation counting. Thymidine incorporation by frozen and thawed cells was normalized relative to values for control, nonfrozen cells.

The results presented in Figure 3 indicate that, as was the case with dimethylsulfoxide in Example 1, the presence of 1-100 µg/ml of THP enhanced the level of cryopreservation provided by glycerol. In contrast, THP at concentrations ≥ 500 µg/ml negated the cryoprotection afforded by glycerol and induced a high degree of damage to the cells.

EXAMPLE 4

KG-1A cells were centrifuged and brought to a density of 5 million cells/ml with DMEM (with 10% FCS). 200 µl

- 16 -

aliquots of the cell suspension were placed in a 12 X 75 mm polypropylene test tubes. To this suspension was added 800 ul of a solution prepared in DMEM and containing the appropriate amount of extracellular cryoprotectant and THP. The cells were frozen by cooling at 3°C/min, stored overnight in liquid nitrogen vapor, and thawed by immersing the rack of test tubes in a 37°C water bath. The cryoprotectant was diluted in the thawed samples by centrifuging the cells, removing 800 ul of the supernatant and replacing it with 800 ul of DMEM. This step was repeated and then triplicate 200 ul samples were removed from each tube and placed in a 96-well microtiter plate. Viability was assessed using the thymidine incorporation assay described in Example 3.

Fig. 4 compares the effect of THP on the viability of KG-1A cells frozen and thawed in the presence of 20% (wt/vol) Ficoll (mean molecular weight = 70 kD) versus in 20% (wt/vol) polyvinylpyrrolidone (PVP; mean molecular weight = 40 kD). THP at levels as low as 5 µg/ml greatly reduced the viability of cells cryopreserved with Ficoll. In contrast, the addition of THP greatly enhanced the degree of cryopreservation afforded by PVP. The latter results indicate that THP can be used to maximize the effectiveness of PVP as a cryoprotectant.

EXAMPLE 5

A transplantable tissue (e.g., heart valve, vein, artery, tendon, ligament, cartilage) would be placed in a solution containing the appropriate mixture of cryoprotectants and thermal hysteresis protein. The tissue would then be frozen. Even for tissue stored in liquid nitrogen or liquid nitrogen vapor, the presence of the thermal hysteresis protein would serve to enhance the level of cryoprotection and hence, the percentage of cells remaining viable after thawing. Also, damage arising due to thawing at suboptimal warming rates would be ameliorated. Moreover, with the addition of the thermal hysteresis

- 17 -

protein, tissue and cell damage, induced by thermal cycling during transportation and/or storage at temperatures higher than -100°C , would be minimized. Therefore, THP would allow one to have more leeway in handling frozen tissue and in the thawing process and still recover the maximum cell viability upon thawing.

EXAMPLE 6

An intact organ (e.g., kidney, liver, heart, pancreas) would be flushed with an appropriate mixture of cryoprotectants and thermal hysteresis protein. The organ would then be cooled until frozen. With the presence of THP it would not be necessary to store the frozen organ at an ultracold temperature, which is usually thought to be needed to prevent damage due to migratory ice recrystallization. Thus, for example, the organ could be cooled to temperatures around -20°C , which would induce extracellular freezing (but not intracellular freezing). At these temperatures, metabolism would be profoundly depressed and the organ could be held for long periods of time. The presence of the THP would prevent the extracellular and intravascular damage that is associated with ice crystal growth. Without the THP, at these high subzero temperatures, the time dependent growth of large ice crystals, at the expense of smaller crystals, would lead to massive damage of the extracellular matrix as well as the endothelial lining of the blood vessels.

EXAMPLE 7

Usefulness of THP during freeze-drying.

Aliquots of cells (e.g., blood cells, plant cells, cultured cell lines) would be mixed with a combination of THP and appropriate lyoprotective compound(s) (e.g., glucose, trehalose, polyanionic compounds, or any of the cryoprotectant materials discussed above, including mixtures thereof). In addition, a set of control samples would be prepared in an identical manner, except that THP would not be present. The lyoprotectants would protect against the damage

- 18 -

induced to cells by freeze-drying (lyophilization), analogously to the protection afforded to cells by cryoprotectants during freeze-thawing. That is, the lyoprotectants would protect the cells from damage arising during freezing and stabilize the cellular elements when the associated water is removed by sublimation.

THP would be present at a level of 1 - 100 $\mu\text{g/ml}$. The samples would be frozen either by direct plunging into liquid nitrogen (i.e., $> 100^\circ\text{C/minute}$ cooling rate) or in a rate-controlled freezing unit ($< 100^\circ\text{C/minute}$). The rate chosen would depend on the cell type and the lyoprotectants. The frozen samples would then be placed in a lyophilizer and subjected to a vacuum to remove the water from the samples.

During this sublimation period the temperature of the samples would rise to above -100°C , and even to a temperature approaching the melting point of water. Migratory recrystallization of ice could occur in the samples in which THP was not present. In contrast, in those samples containing THP, migratory recrystallization, and the concomitant cell damage, would be inhibited.

After the samples had reached the desired level of dryness (e.g., about 1% residual moisture) they would be removed from the lyophilizer and rehydrated by the addition of water or other suitable aqueous medium. Those samples containing THP would have a higher level of cell survival than those freeze-dried without THP. In this case the THP would be protecting the cells from damage due to ice crystal growth during the sublimation phase of the freeze-drying process. This is analogous to the protection provided by the THP during the thawing phase of a freeze-thawing process. Therefore, the overall effect of the THP would be to enhance the degree of protection afforded by the lyoprotectants.

This process could also be used for preserving the functional integrity of other membrane enclosed, bound, or encapsulated material during freeze-drying, e.g., material

- 19 -

enclosed, bound, or encapsulated by a phospholipid membrane. Certain nonlimiting examples of such material include liposomes, organelles, subcellular organelles, artificial vesicles, viruses, and immunological material.

It will be apparent to those skilled in the art that various modifications and variations can be made in the processes and products of the present invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

SUBSTITUTE SHEET

- 20 -

WHAT IS CLAIMED IS:

1. A method for enhancing survival of biological material during cryopreservation comprising freezing and subsequently thawing biological material in the presence of at least one cryoprotectant and a thermal hysteresis peptide in an amount effective for enhancing viability of the biological material.
2. A method as in claim 1, wherein said freezing and thawing include using at least one cryoprotectant capable of penetrating into an intracellular area of the biological material.
3. A method as in claim 1, wherein said freezing and thawing include using at least one cryoprotectant which does not penetrate into an intracellular area of the biological material.
4. A method as in claim 1, wherein said freezing and thawing include using a thermal hysteresis peptide having the following amino acid sequence: (NH₂) ASP-THR-ALA-SER-ASP-ALA-ALA-ALA-ALA-ALA-ALA-LEU-THR-ALA-ALA-ASN-ALA-LYS-ALA-ALA-ALA-GLU-LEU-THR-ALA-ALA-ASN-ALA-ALA-ALA-ALA-ALA-ALA-THR-ALA-ARG (COOH).
5. A method as in claim 1, wherein said freezing and thawing include using a thermal hysteresis peptide selected from the group consisting of a glycopeptide having a molecular weight of about 2,600 to 37,000 having high proportions of alanine and threonine, a peptide having a molecular weight of about 3,300 to 4,000 having a high alanine content, a peptide having a molecular weight of about 9,900 containing cysteine and a peptide having a molecular weight of about 6,000 being neither alanine rich nor cysteine rich.
6. A method as in claim 1, wherein said freezing and thawing includes using at least one cryoprotectant selected from the group consisting of dimethylsulfoxide, glycerol, ethylene glycol, propane diols, butane diols, amides,

SUBSTITUTE SHEET

- 21 -

hydroxethyl starch, dextrans, polyethylene glycol, FICOLL, polyvinyl-pyrrolidone, trehalose, maltose, glucose, sorbitol, proline, glutamate, mannitol and betaine.

7. A method as in claim 1, wherein said freezing and thawing includes using at least one cryoprotectant selected from the group consisting of dimethylsulfoxide, glycerol, ethyleneglycol, hydroxethyl starch, dextrans, polyethylene glycol, polyvinyl-pyrrolidone, trehalose, sorbitol, proline and glutamate.

8. A method as in claim 1, wherein said freezing and thawing includes using thermal hysteresis peptide in an amount no greater than 200 $\mu\text{g/ml}$.

9. A method as in claim 1, wherein said freezing and thawing includes using thermal hysteresis peptide in an amount of about 1-100 $\mu\text{g/ml}$.

10. A method as in claim 1, wherein said freezing and thawing include using isolated cells comprising at least one of lymphocytes, islets of Langerhans, bone marrow cells, sperm and ova as the biological material.

11. A method as in claims 1, wherein said freezing and thawing includes using an organ or organ tissue as the biological material.

12. A method as in claim 1, wherein said freezing and thawing includes using transplantable tissue.

13. A method as in claim 12, wherein said transplantable tissue includes at least one of the group heart valve, vein, artery, tendon, ligament and cartilage.

14. A method as in claim 1, wherein said freezing and thawing includes using noncellular biological materials comprising at least one of enzymes, liposomes, blood platelets, encapsulated proteins, isolated proteins, cell organelles and nucleic acid as the biological material.

15. A method as in claim 1, wherein said freezing and thawing of biological material in the presence of at least one cryoprotectant and a thermal hysteresis peptide enhances

SUBSTITUTE SHEET

- 22 -

the viability of the biological material at warming rates that can damage the biological material, in the absence of thermal hysteresis peptide.

16. A method as in claim 15, wherein said thawing includes thawing in air at approximately room temperature.

17. A method as in claim 1, wherein said freezing includes long-term storage at temperatures greater than -100°C.

18. A composition for enhancing survival of biological material during cryopreservation and thawing comprising at least one cryoprotectant and a thermal hysteresis peptide in an amount effective for enhancing viability of the biological material.

19. A composition as in claim 18, wherein said at least one cryoprotectant is capable of penetrating into an intracellular area of the biological material.

20. A composition as in claim 18, wherein said at least one cryoprotectant does not penetrate into an intracellular area of the biological material.

21. A composition as in claim 18, wherein said thermal hysteresis peptide has the following amino acid sequence: (NH₂) ASP-THR-ALA-SER-ASP-ALA-ALA-ALA-ALA-ALA-ALA-LEU-THR-ALA-ALA-ASN-ALA-LYS-ALA-ALA-ALA-GLU-LEU-THR-ALA-ALA-ASN-ALA-ALA-ALA-ALA-ALA-ALA-ALA-THR-ALA-ARG (COOH).

22. A composition as in claim 18, wherein said cryoprotectant is selected from at least one of the group consisting of dimethylsulfoxide, glycerol, ethylene glycol, propane diols, butane diols, amides, hydroxethyl starch, dextrans, polyethylene glycol, FICOLL, polyvinyl-pyrrolidone, trehalose, maltose, glucose, sorbitol, proline, glutamate, mannitol and betaine.

23. A composition as in claim 18, wherein said thermal hysteresis peptide is in an amount no greater than 200 µg/ml.

SUBSTITUTE SHEET

- 23 -

24. A composition as in claim 18, wherein said thermal hysteresis peptide is in an amount of about 1-100 $\mu\text{g/ml}$.

25. A composition for enhancing survival during cryopreservation and thawing of at least one of isolated cells, tissues, organ and organ tissue comprising at least one cryoprotectant and a thermal hysteresis peptide in an amount effective to enhance viability of the isolated cells, tissues, organ or organ tissue.

26. A composition for enhancing survival during cryopreservation and thawing of noncellular biological materials including at least one of enzymes, liposomes, blood platelets, encapsulated proteins, isolated proteins, cell organelles and nucleic acid comprising at least one cryoprotectant and the thermal hysteresis peptide in an amount effective to enhance viability of the enzymes, liposomes, blood platelets, encapsulated proteins, isolated proteins, cell organelles or nucleic acid.

27. A method for preserving the functional integrity of membrane enclosed or bound material during freeze-drying comprising freeze-drying the membrane enclosed or bound material in the presence of at least one lyoprotectant and a thermal hysteresis peptide in an amount effective for preserving the functional integrity of the membrane enclosed or bound material.

SUBSTITUTE SHEET

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC(3) A01N 1/00, 1/02; A61K 37/00, 37/02; C07K 7/10
 US: 435/1,2; 514/12; 530/324

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
US	435/1,2; 530/324; 514/12

Documentation Searched other than Minimum Documentation
 to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	PROC. R. SOC. LOND B. Vol 234. issued 1988. B. Rubinsky et al., "A Mathematical Model for the Freezing Process in Biological Tissue", pages 343-358, see entire document.	1.11-13. 16-18
Y	D.E. PEGG, "THE BIOPHYSICS OF ORGAN PRESERVATION", published 1987 by Plenum, pages 117-140, see entire document.	1.11-13. 16-18
X Y	ANN. REV. PHYSIOL., Vol. 45. issued 1983, A.L. De Vries, "Antifreeze Peptides and Glycopeptides in Cold Water Fishes", pages 245-260, see entire document.	1-5,8-10 <u>15.20-27</u> 1-5,8-10 15.20-27

* Special categories of cited documents: ¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier document but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

01 May 1991

21 JUN 1991

International Searching Authority

Signature of Authorized Officer

ISA/USA

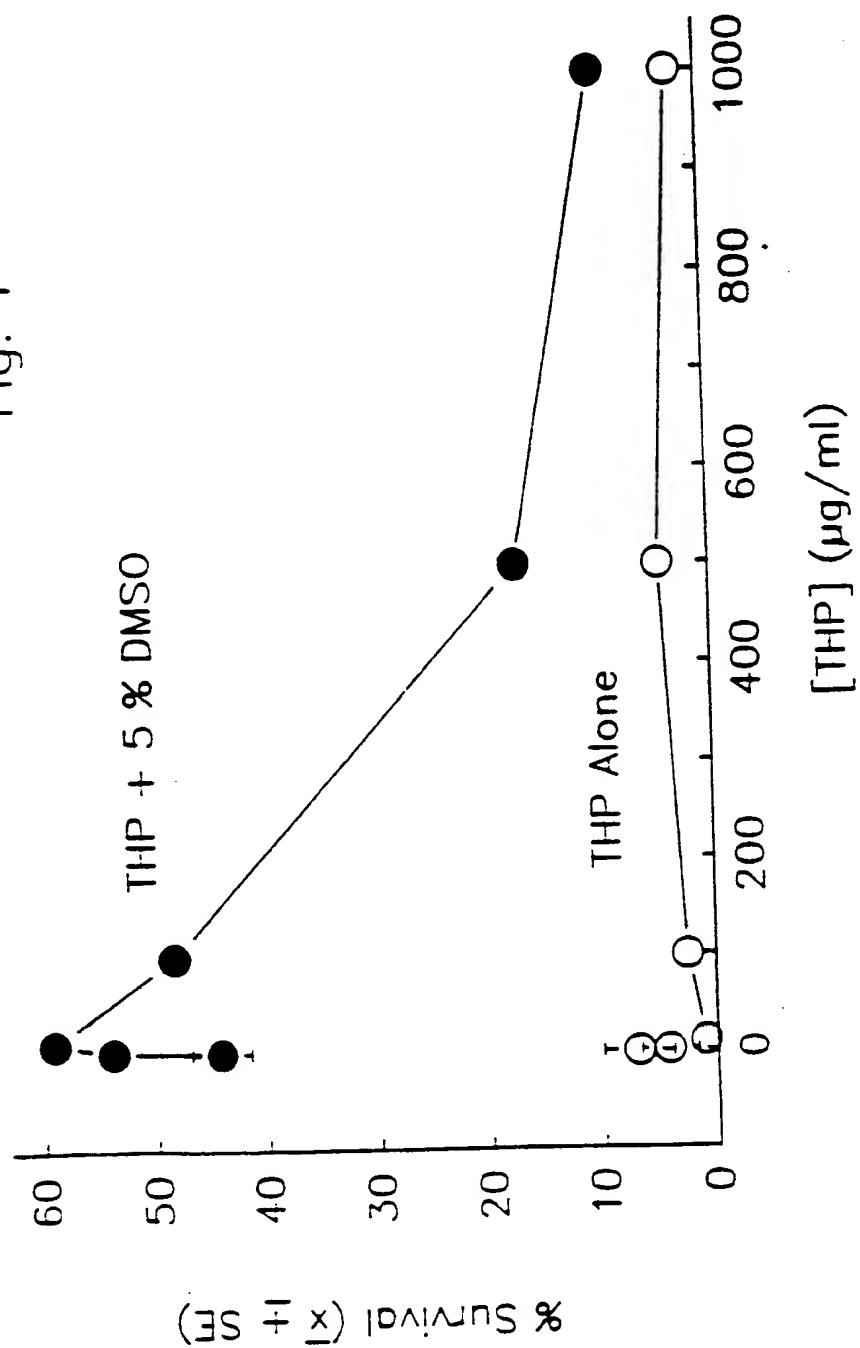
Jon P. Weber

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
<u>X</u> Y	ANN. REV. PHYSIOL., Vol. 45, issued 1983. J. Duman et al., "The Role of Hemolymph Proteins in the Cold Tolerance of Insects", pages 261-270. see entire document.	<u>1-27</u> 1-27
<u>X</u> Y	CRYOBIOLOGY, Vol. 23, issued 1986, C.A. Knight et al., "Inhibition of Recrystallization of Ice by Insect Thermal Hysteresis Proteins: A Possible Cryoprotective Role", pages 256-262, see entire document, especially materials and methods.	<u>1-27</u> 1-27
<u>X</u> Y	CRYOBIOLOGY, Vol. 26, issued 1989, G.L. De Antoni et al., "Trehalose, a Cryoprotectant for Lactobacillus bulgaricus", pages 149-153, see introduction.	2-3.6-7 <u>19-20</u> 2-3.6-7 19-20
Y	CRYOBIOLOGY, Vol. 27, issued February 1990, D.R. MacFarlane et al., "Recent Insights on the Role of Cryoprotective Agents in Vitrification", pages 345-358, see entire document.	1-27
<u>X</u> Y	KARROW, ET AL., "ORGAN PRESERVATION FOR TRANSPLANTATION," published 1974 by Little, Brown and Company (Boston), pages 86-107, see entire document.	<u>1-27</u> 1-27

1/4

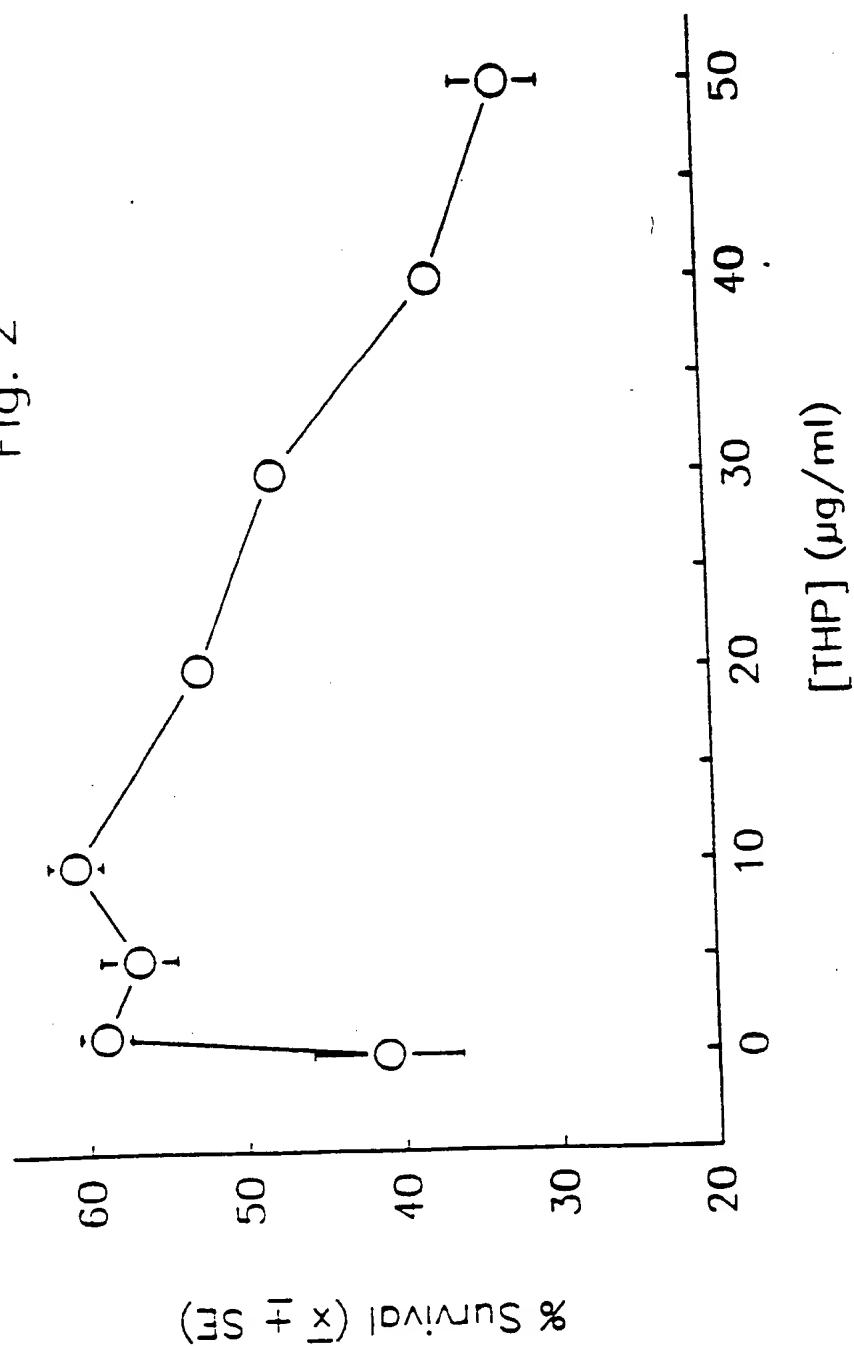
Fig. 1



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2/4

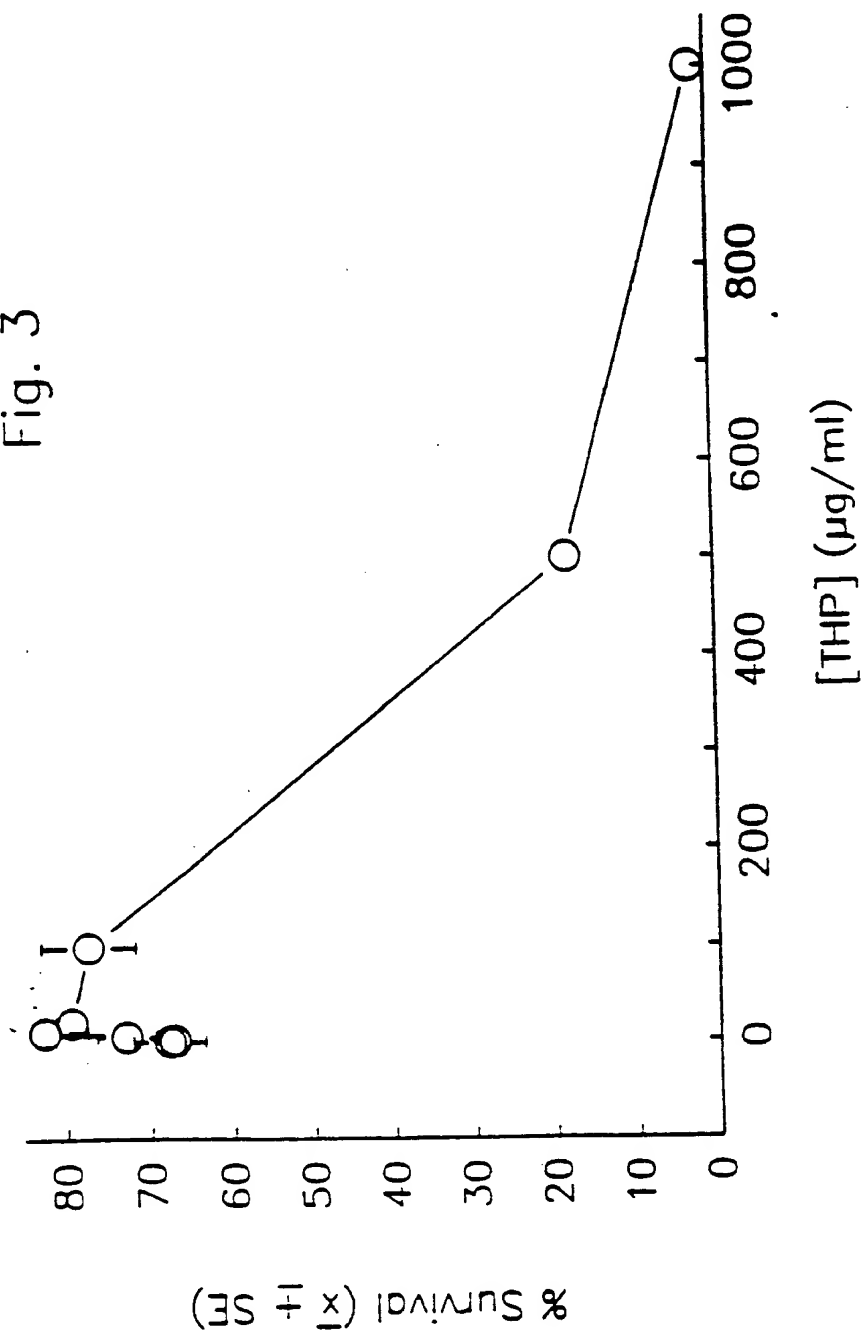
Fig. 2



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3/4

Fig. 3



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4/4

